

REMARKS

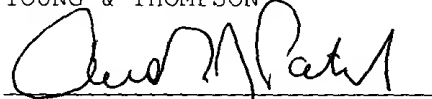
The specification has been amended to conform to the accompanying Sequence Listing.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "Version with markings to show changes made."

Respectfully submitted,

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By



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Version with markings to show changes made

IN THE SPECIFICATION:

Paragraph beginning at line 10 and ending at line 20 of page 21 has been amended as follows:

As example of oligonucleotides, one may cite the following:

GEM 91

phosphorothioate (X = S) oligonucleotide i = 25 (SEQ ID

NO: 1)

CTC TCG CAC CCA TCT CTC TCC TTC T

complementary to the AUG initiation site of gag HIV-1 gene

ISIS 1939

phosphorothioate (X = S) oligonucleotide i = 19 (SEQ ID

NO: 2)

CCC CCA CCA CTT CCC CTC T

complementary to the 3' non coding region of ICAM-1 mRNA.--

Paragraph beginning at line 25 of page 25 has been amended as follows:

As an example of the specific TFO is an oligonucleotide 5'-A<sub>4</sub>GA<sub>4</sub>G<sub>6</sub>A-3' (SEQ ID NO: 6) directed against the polypurine track (PPT) in the NEF-HIV-1 gene.

Paragraph beginning at line 1 of page 27 has been amended as follows:

A) Cells are incubated for 4 h at 37°C with 1 µM fluorescein-labelled peptide (F-S-CGEEDTSEKDEL) (SEQ ID NO:3) in the absence or in the presence of histidylated oligolysine. Cells are fixed with 2 % of p-formaldehyde, washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent. Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope.

Paragraph beginning at line 10 of page 27 has been amended as follows:

B) Dendritic cells are incubated for 4 h at 37°C with 1 µM c-myc epitope peptide (SMEQKLISEEDLNFELEA) (SEQ ID NO:4) in the absence or in the presence of histidylated oligolysine. Cells are fixed with 2 % of p-formaldehyde in the presence of 0.5 % saponine, washed and then incubated for 1 h with anti c-myc epitope monoclonal antibody (9E10) in PBS containing [containing] 10 mg/ml BSA and 0.1% saponin. Cells are washed and further incubated for 1 h in the presence of fluorescein-labelled anti-mouse IgG F(ab)' fragments in PBS containing [containing] 10 mg/ml BSA and 0.1% saponin. Cells are washed and mounted on slides in a PBS/glycerol mixture (1:1 v/v) containing 10 mg/ml DABCO (1,4 diazobicyclo-(2,2,2)-octane) as antifading agent.

Cells are analyzed with a confocal microscope imaging system (MRC-1024, Bio-Rad) equipped with a Nikon Optiphot epifluorescence microscope.

Paragraph beginning at line 9 of page 28 has been amended as follows:

Dendritic cells were incubated for 4 h at 37°C with the nonadecapeptide (185-203) from the C-terminal part of the HIV-1 Nef protein containing the nonapeptide (190-198) (AFHHVAREL) (SEQ ID NO:5) in the absence or in the presence of an histidylated oligolysine. Cells are washed and further incubated for 24 h at 37°C in the absence of peptide and histidylated oligolysine. MHC class I presentation of peptide antigen was evaluated by Cr<sup>51</sup> cytotoxic assay by using a CTL clone sensible to the peptide. DCs were labeled with Cr<sup>51</sup> (target cells : T) and then incubated at 37°C for 4 h in the presence of the CTL clone (effector cells : E) at E/T ratios ranged from 1 to 100. The supernatants are collected and the radioactivity in the supernatant was recorded.

The % of specific Cr<sup>51</sup> release is calculated according to  $(A_{NS} - A_S)/A_{NS} \times 100$  where  $A_{NS}$  and  $A_S$  are the radioactivity in supernatant dilutions of dendritic cells incubated in the absence and the presence of CTL cells, respectively.

Paragraph beginning at line 20 of page 30 has been amended as follows:

Figure 1 shows the activity of GEM-91, an antisense phosphorothioate oligonucleotide (PS-ODN) (CTC TCG CAC CCA TCT

CTC TCC TTC T) (SEQ ID NO:1) complementary to the AUG initiation site of gag HIV-1 gene. The effect of histidylated oligolysines was evaluated by using pRET-Luc cells (a rabbit smooth muscle cell line). These cells produce endogenous luciferase under the control of the human phosphoglycerate kinase promoter and the luciferase gene sequence around the AUG codon was replaced by the initiator AUG codon and several downstream codons of gagHIV-1 gene. The results showed that the activity of GEM-91 ( $IC_{50} > 5 \mu M$ ) was increased more than 10 times in the presence of  $20 \mu M$  HoK2 ( $IC_{50} 0.25 \mu M$ ). Whilst, no significant inhibition was obtained in the presence of HoK3 in which the  $\alpha-NH_2$  histidyl residues were acetylated, suggesting that interactions between ODN and histidylated oligolysines were involved. Prêt-Luc cells, seeded onto 24-well plates ( $2 \times 10^5$  cells/well), were treated for 4 h at  $37^\circ C$  in DMEM supplemented with 2 % FBS containing various concentrations of GEM-91, (■) in the absence of histidylated oligolysine, (●) in the presence of  $20 \mu M$  HoK2 or (○) in the presence of  $20 \mu M$  HoK3. HoK2 and HoK3 are histidylated oligolysines prepared as described in the following text. Then, FBS was raised to 6 % and cells were further incubated for 18 h. Luciferase gene expression was measured by recording luminescence for 4 s. The percentage of luciferase inhibition was calculated by using ,  $[(RLU^{ODN}-RLU)/RLU] \times 100$  where  $RLU^{ODN}$  and RLU were the luciferase activity into cell lysates of cells incubated in the absence and in the presence of ODN, respectively. Results shown

typical of experiments carried out in triplicate and repeated at least twice. Data are means  $\pm$  standard deviation.

Paragraph beginning at line 14 of page 31 has been amended as follows:

Figure 2 shows the inhibitory effect of TNF- $\alpha$  induced ICAM-1 expression by ISIS 1939 (CCCCCACCACCTCCCCCTCT) (SEQ ID NO:2), an antisense phosphorothioate oligonucleotide (PS-ODN) targeted to the 3' non-coding region of ICAM-1 mRNA. The results showed that TNF- $\alpha$  induced ICAM-1 expression was inhibited by ISIS 1939 in the presence of 20  $\mu$ M of histidylated oligolysines. HoK2 (IC<sub>50</sub> of 0.25  $\mu$ M) appeared to be more efficient than HoK1 (IC<sub>50</sub> of 0.5  $\mu$ M) probably because HoK2 bore less histidyl residues than HoK1 (15 versus 12). The inhibition was very low in the absence of histidylated oligolysines even up to 1  $\mu$ M ODN (20 % inhibition). A549 cells (ATCC CCL 185, Rockville, MD) were plated onto 96-wells microtiter plates (10<sup>4</sup> cells/well). The day after, culture medium was removed and cells were washed. Cells were incubated at 37°C for 4 h in 100  $\mu$ l DMEM serum-free medium containing ISIS 1939 ODN either in the absence (■) or in the presence of 20  $\mu$ M (●) HoK1 or (□) HoK2. HoK2 and HoK3 are histidylated oligolysines prepared as described in the following text. One volume of fresh medium containing 10 ng/ml TNF- $\alpha$  was added and cells were further incubated for 18 h. ICAM-1 expression was quantified by ELISA using anti-ICAM-1 antibodies. Cells were washed 3 times with 200  $\mu$ l of PBS and fixed for 20 min

at room temperature in PBS containing 20 mg/ml paraformaldehyde. Then, cells were incubated for 90 min at 37°C with anti-ICAM 1 mouse antibody (Becton Dickinson) diluted 20 times in PBS containing 20 mg/ml BSA. Cells were washed 3 times with PBS and then incubated for 1 h at 37°C with an anti-mouse horseradish peroxidase conjugate (Becton Dickinson) diluted 2000 times in PBS containing 20 mg/ml BSA. After 3 [whashes] washes, the peroxidase activity was assessed by using 100 µl of o-phenylenediamine dihydrochloride peroxidase substrate tablet set (Sigma). After X min incubation at 37°C, the reaction was stopped by adding 25 µl of 3 N H<sub>2</sub>SO<sub>4</sub> and the absorbance read at 492 nm. All calculations were made relative to untreated controls in the absence or in the presence of TNF-α. The percentage of TNF-α-induced expression of ICAM-1 was calculated as follows :  $[A_{\text{TNF-}\alpha}^{\text{ODN}} - A_0] / (A_{\text{TNF-}\alpha} - A_0) \times 100$  where  $A_{\text{TNF-}\alpha}^{\text{ODN}}$  was the absorbance of ODN treated and cytokine-induced cells,  $A_0$  the absorbance of cells incubated without ODN and TNF-α, and  $A_{\text{TNF-}\alpha}$  the absorbance of cytokine-induced cells incubated without ODN. Results shown are typical of experiments carried out in triplicate and repeated at least twice. Data are means ± standard deviation of the percentage of control ICAM-1 expression induced by TNF-α.

Heading appearing at line 2 of page 38 has been amended as follows:

Example 7 : Preparation of (SEQ ID NO:7) (K(His)-KL(His)-L)<sub>7</sub>.

Paragraph beginning at line 3 of page 38 has been amended as follows:

An oligomer (K(His)-KL(His)-L)<sub>7</sub>, (SEQ ID NO:8) can be entirely synthesised by using the above Lys (His) synthon and Fmoc Leu on a Applied Biosystems 433A synthesizer with conductimetric monitoring by using Fmoc-protected amino acids. Lys(His) synthons and Leu are coupled by the HBTU activation method. The oligomer (K(His)-KL(His)-L)<sub>7</sub> is cleaved from the resin and side chain protecting Boc groups are removed with a trifluoroacetic acid/water mixture (50% : 50% ; v/v) for 3 h at room temperature. The polymer is precipitated with isopropanol and collected by centrifugation. The oligomer is washed three times with isopropanol, resuspended in distilled water and freeze-dried.

Heading appearing at line 12 of page 38 has been amended as follows:

Example 8 : Preparation of (SEQ ID NO:9) (K(His)-L-K(His))<sub>7</sub>.

Paragraph beginning at line 13 of page 38 has been amended as follows:

A oligomer (K(His)-L-K(His))<sub>7</sub>, (SEQ ID NO:9) can be entirely synthesised by using the above Lys (His) synthon and Fmoc Leu on a Applied Biosystems 433A synthesizer with



conductimetric monitoring by using Fmoc-protected amino acids. Lys(His) synthons and Leu are coupled by the HBTU activation method. The oligomer is cleaved from the resin and side chain protecting Boc groups are removed with a trifluoroacetic acid/water mixture (50% : 50% ; v/v) for 3 h at room temperature. The oligomer is precipitated with isopropanol and collected by centrifugation. The oligomer is washed three times with isopropanol, resuspended in distilled water and freeze-dried.